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Screening for basic drugs in equine urine using direct-injection differential-gradient LC–LC coupled to hybrid tandem MS/MS

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Abstract

A rapid, selective and robust direct-injection LC/hybrid tandem MS method has been developed for simultaneous screening of more than 250 basic drugs in the supernatant of enzyme hydrolysed equine urine. Analytes, trapped using a short HLB® extraction column, are refocused and separated on a Sunfire® C_{18} analytical column using a controlled differential gradient generated by proportional dilution of the first column's eluent with water. Independent data acquisition (IDA) was configured to trigger a sensitive enhanced product ion (EPI) scan when a multiple reaction monitoring (MRM) survey scan signal exceeded the defined criteria. The decision on whether or not to report a sample as a positive result was based upon both the presence of a MRM response within the correct retention time range and a qualitative match between the EPI spectrum obtained and the corresponding reference standard. Ninety seven percent of the drugs targeted by this method met our detection criteria when spiked into urine at 100 ng/ml; 199 were found at 10 ng/ml, 83 at 1 ng/ml and 4 at 0.1 ng/ml.

Keywords: Drug testing; Direct injection; Tandem hybrid mass spectrometry; Differential LC–LC gradient

1. Introduction

Pharmaceutical companies are introducing new medicines at an ever-increasing rate just to try to maintain their share of the fiercely competitive international marketplace and this has led to a large increase in the number of easily available drugs with high potential for affecting racehorse performance. To make matters worse rogue chemists are trying to find ways of evading doping control programmes by producing so-called "designer drugs" through slight chemical modification to compounds with well-known pharmacological activity. Abuse of these drugs will eventually be uncovered, take for example tetrahydrogestrinol, and this will add to the already prodigious list of screening targets that horseracing and other drug screening laboratories should have the capability to detect. Consequently, these trends mean that laboratories must continue to expand their drug testing repertoire and this should be done at a rate commensurate with the rapid expansion of the number of potential doping agents.

Another factor shaping horseracing doping surveillance programmes is that some of the latest drugs are much more potent than the older analogues from the same pharmacological class. This has led laboratories to increasingly adopt methodology with the specificity and selectivity to detect low levels of drugs and/or their metabolites in equine urine and blood. Hence, when a suitable immunoassay is not available, a commonly used approach is off-line sample extraction followed by hyphenated instrumental analysis targeting a defined class of drugs. The underlying basis for the selection of the particular class of the analytical targets can be the presence of an important chemical moiety, like a quaternary amine group, or the principal pharmacological property of the drugs. Whilst this analytical approach should not automatically exclude the detection of drugs from other classes, in practice the selection of optimised extraction, preparation and analytical steps during method development restricts the application of the screen to the identification of a relatively small (usually < 20) number of targets. Even a brief survey of the current scientific literature will reveal a large number of examples that are premised upon this analytical philosophy (e.g. [1]). However, adopting a strategy of using narrowly focused analyses in preference to relatively broad based techniques is

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inefficient, as ultimately a larger number of extraction methods will be required to provide the desired scope of coverage demanded by the customer. This would mean that the minimum sample volume needed to screen for a reasonably large proportion of the potential doping agents that are currently available would probably exceed the volume that is typically supplied to the laboratory for analysis and, moreover, the demand for labour, space and equipment to complete the screening in this manner would be significant. For these reasons, a shift in emphasis from using narrow, class-based, analyses towards adopting sensitive methodologies capable of detecting a wider range of drugs during a single analysis has become evident (e.g. [2,3]).

Methods have been published that improve the ratio of drugs targeted to volume of sample consumed. For example, it has been proposed [4] that serial processing of equine urine on speciality solid-phase extraction (SPE) sorbents will allow speciation of a single sample aliquot into many different chemical classes of extract that are suitable for instrumental analyses. However, because of widely divergent nature of the chemicals involved, stoichiometric recovery of all types of drugs at every stage along the extraction path is unlikely and accumulated losses incurred over multiple extractions/manipulation steps will give an unacceptable final percentage recovery for some compounds. For example, the same authors reported [5] that the recovery of furosemide after two out of four extraction and elution steps was only 19%. Ultimately, the laboratory will require a number of additional extraction methods to cope with those drugs that are not amenable to serial extraction and they have recently published [6] a separate extraction method for quaternary amines and anabolic steroids. The introduction of additional (parallel) extraction methods negates the sample conservation benefit obtained by serial processing.

Another tactic that has been used to reduce sample consumption is integrating the sample preparation and chromatographic separation steps. The technique is often referred to as "direct-injection" because a small volume of sample, that has undergone limited or no pre-extraction treatment, is introduced directly onto the LC column. This also provides a significant time advantage over off-line sample pre-extraction methods where separate extraction/purification, evaporation and reconstitution tasks are undertaken before the sample is ready for instrumental analysis.

We adopted a direct injection LC–MS/MS set-up when developing a new screening method aimed at detecting a wide variety of drugs in equine urine, as this had previously been shown to be successful [7–23].

The LC was coupled to an Applied Biosystems 4000 QTRAP fitted with a Duospray® source capable of providing both TurboIonSpray® (TIS) and heated nebulizer® (HN) atmospheric pressure ionisation. This mass spectrometer was selected because of its software switchable ability to acquire product ion spectra in the sensitive linear ion trapping (LIT) mode whenever a multiple reaction monitoring (MRM) peak exceeded our defined IDA criteria. This instrument also has the capacity to perform a large number of survey scans within a short time frame because it has a linear acceleration collision cell (LINAC) that enables ions to be transported through the system very rapidly [24]. A screen for 300 drugs in urine has been reported [24] using a 4000 QTRAP configured to undertake very short dwell time (<10 ms) MRM experiments. In our case, we have shown that this mass spectrometer can perform MRM (10 ms) and dependent enhanced product ion (EPI) (maximum fill time 20 ms) scanning experiments to analyse centrifuged enzyme-hydrolysed samples for more than 250 drug targets. This was achieved with sufficient sensitivity to permit detection of drugs in positive equine urine samples.

2. Experimental

2.1. Materials and reagents

The 257 reference standards used were obtained from various suppliers shown by number in column 2 of Table 1. The number correspond with: (1) Sigma–Aldrich (Singapore); (2) United States Pharmacopeia (Rockville, MD, USA); (3) National Metrology Institute (Sydney, Australia); (4) Fluka (Singapore); (5) Neogen Corporation (Lexington, KY, USA); (6) gift from Racing Analytical Services Limited (Melbourne, Australia); (7) gift from the Hong Kong Jockey Club (Hong Kong, China) and (8) the proprietary medicine's manufacturer. Water used to prepare the aqueous mobile phase was purified using a Millipore Elix® pre-treatment system to feed a MilliQ® reverse osmosis water purification unit. The total organic carbon in the purified water was less than 10 ppb and resistance was greater than 18 megaohmn. Acetonitrile and methanol were HPLC grade from Fischer Scientific (Pittsburgh, PA, USA) and formic acid (>98%) was Cica® grade from Kanto Kaguka (Singapore). *Helix pomatia* β-glucuronidase/aryl sulphatase enzyme was from Roche Diagnostics Asia Pacific (Singapore). The 25% ammonia solution and ammonium acetate GR were obtained from Merck PTY Ltd. (Singapore). Organic solvents and aqueous buffers were passed through a 0.2 μm Whatman (Maidstone, England) filter made of either cellulose nitrate (aqueous) or PTFE (organic) material before being used as LC mobile phases.

2.2. Instrumentation and chromatographic conditions

An Agilent (Singapore) HP1100 series LC consisting of a G1316A column oven with 6-port valve, G1311A quaternary, G1310A isocratic pump plus a G1367A autosampler retrofitted with a multi-draw option kit that extends the maximum injection volume to 500 μl, was connected to an Applied Biosystems (Singapore) MDS Sciex 4000 Q Trap hybrid tandem mass spectrometer operating under Analyst 1.4.1. The Shimadzu (Singapore) LC6A was used as the second isocratic pump. Two (Valco Instruments Company, Houston, TX, USA) 10-port valves (040–0811 V) with microelectric valve actuators, controlled by Analyst 1.4.1 software, were mounted next to the DuoSpray® TIS and HN dual spray source. The (Waters

Table 1

No.	Drug name	From sup.	MRM transition parameters				Rt (min)	Detected at spiked level? (ng/ml)[a]			
			Q1	Q3	DP	CE		0.1	1	10	100
(a) MRM acquisition parameters and the limit of detection achieved for the target analytes screened using MRM exp 1											
1	1,1-Dimethylbiguanide	1	130.2	85	46	20	4.39				N
2	2-(1-Hydroxyethyl)promazine-sulphoxide	5	345.3	243.0	70	39	8.20			Y	Y
3	2-Amino-4-picoline	1	109.0	92.0	73	29	5.21			Y	Y
4	3,4-Methylenedioxyamphetamine	3	180.2	135.0	29	26	8.00		Y	Y	Y
5	3,4-Methylenedioxyethamphetamine	3	208.1	163.0	53	18	8.17		Y	Y	Y
6	4-Methyl-2,5-dimethoxy amphetamine	2	210.2	165.1	42	26	8.50			Y	Y
7	Acebutolol	1	337.0	116.1	76	40	8.24		Y	Y	Y
8	Acepromazine	1	327.1	58.2	71	40	8.96			Y	Y
9	Adiphenine	1	312.2	239.2	74	26	8.96			Y	Y
10	Alfentanil	2	417.4	314.2	83	40	8.77		Y	Y	Y
11	Alphaprodine	2	262.4	188.2	67	40	8.61				Y
12	Alprazolam	1	309.1	281.1	62	36	10.17		Y	Y	Y
13	Alprenolol	1	250.2	116.1	60	25	8.77			Y	Y
14	Ambroxol	1	379.0	263.9	60	29	8.41			Y	Y
15	Amiloride	1	230.1	171.1	68	28	7.71			Y	Y
16	Amlodipine	8	409.2	238.0	56	40	9.13				Y
17	Amoxapine	2	314.0	271.1	76	33	8.88			Y	Y
18	Amphetamine	3	136.1	91.0	65	30	7.91				Y
19	Anileridine	2	353.2	120.1	80	40	8.32		Y	Y	Y
20	Anisotropine methylbromide	1	282.2	138.2	89	35	8.92		Y	Y	Y
21	Apomorphine	1	268.1	237.2	69	25	8.06		Y	Y	Y
22	Atracurium	6	464.3	601.4	76	40	8.68				Y
23	Azaperol	6	330.1	121.2	82	40	7.99		Y	Y	Y
24	Baclofen	1	214.1	179.1	44	20	7.93			Y	Y
25	Benazepril	2	425.2	351.4	66	40	9.51		Y	Y	Y
26	Benzocaine	2	166.1	138.1	37	15	10.20			Y	Y
27	Benzphetamine	2	240.2	91.0	48	39	8.76		Y	Y	Y
28	Benztropine	1	308.0	167.1	75	40	9.21		Y	Y	Y
29	Benzydamine	1	310.2	265.2	74	25	9.00				Y
30	Betaxolol	2	308.2	116.1	85	28	8.76			Y	Y
31	Biperiden	2	312.2	98.1	77	35	8.94		Y	Y	Y
32	Bisoprolol	2	326.2	116.1	96	40	8.59			Y	Y
33	Boldine	1	328.2	297.2	75	24	8.02	Y	Y	Y	Y
34	Bromazepam	8	316.2	182.2	86	45	9.76			Y	Y
35	Bromhexine	1	377.0	114.2	37	28	9.09		Y	Y	Y
36	Bromocriptine	2	654.3	301.1	91	49	9.21				Y
37	Bromopride	1	346.1	273.0	57	32	8.20		Y	Y	Y
38	Buflomedil	1	307.7	237.1	55	40	8.41			Y	Y
39	Bupivacaine	1	289.0	140.2	65	40	8.67		Y	Y	Y
40	Bupropion	1	240.2	184.2	49	20	8.49		Y	Y	Y
41	Butacaine	2	307.2	178.1	75	30	8.76		Y	Y	Y
42	Butaclamol	1	362.2	234.2	66	52	9.07			Y	Y
43	Butamben	2	194.2	120.1	45	31	11.07				Y
44	Caffeine	1	195.0	138.0	56	27	8.50				Y
45	Carbamazepine	1	237.0	194.1	67	32	10.15		Y	Y	Y
46	Cathinone	3	150.1	117.2	51	33	7.75			Y	Y
47	Chlordiazepoxide	2	300.2	227.1	65	40	8.76		Y	Y	Y
48	Chlorpheniramine	2	277.2	232.0	44	40	8.50				Y
49	Chlorprothixene	1	316.2	231.1	67	40	9.32			Y	Y
50	Cilazapril	8	418.4	211.0	106	40	9.07		Y	Y	Y
51	Cimetidine	1	253.1	159.1	45	25	7.62			Y	Y
52	Cinchonidine	1	295.2	168.2	94	43	7.89				Y
53	Cinnarizine	1	369.1	167.1	58	33	9.48		Y	Y	Y
54	Citalopram	1	325.0	262.2	79	29	8.90			Y	Y
55	Clemizole	1	325.8	84.0	68	40	8.99			Y	Y
56	Clenbuterol	1	277.1	203.0	44	24	8.29			Y	Y
57	Clidinium	1	352.1	142.1	76	40	8.74			Y	Y
58	Clobazam	1	301.0	259.1	65	29	10.70			Y	Y
59	Clobenzorex	3	262.1	91.0	56	40	8.91			Y	Y
60	Clopamide	1	346.1	250.0	69	36	9.57			Y	Y

Table 1 (*Continued*)

No.	Drug name	From sup.	MRM transition parameters				Rt (min)	Detected at spiked level? (ng/ml)[a]			
			Q1	Q3	DP	CE		0.1	1	10	100
61	Clozapine	1	327.2	270.0	67	40	8.61			Y	Y
62	Codeine-d3[b]	3	303.1	215.1	83	33	7.78	Y	Y	Y	Y
63	Cotinine	1	177.1	80.0	77	34	7.42		Y	Y	Y
64	Cyclobenzaprine	2	276.0	191.1	21	28	9.15			Y	Y
65	Decamethonium bromide	2	129.1	198.2	56	20	7.58			Y	Y
66	Delorazepam	1	305.1	241.1	94	37	10.71			Y	Y
67	Dembrexine	7	380.1	265.0	63	39	8.28			Y	Y
68	Demecarium bromide	2	279.1	292.2	83	28	8.74				Y
69	Desipramine	2	267.0	72.1	42	28	9.07			Y	Y
70	Dextromethorphan	1	272.0	215.2	112	33	8.83			Y	Y
71	Dextrorphan	1	258.1	201.1	88	30	8.29			Y	Y
72	Diazepam	1	285.3	257.3	69	33	10.87			Y	Y
73	Dibucaine	2	344.2	271.2	86	32	9.20			Y	Y
74	Dichloralphenazone	2	189.0	147.1	75	31	8.99				Y
75	Diethylpropion	2	206.0	105.0	72	34	7.87			Y	Y
76	Dihyrocodeine	2	302.1	199.0	78	46	7.84			Y	Y
77	Dilitiazem	1	415.1	178.0	55	40	8.90		Y	Y	Y
78	Dimidium	1	301.2	285.2	112	58	8.45			Y	Y
79	Diphenhydramine	1	256.2	167.0	35	21	8.92		Y	Y	Y
80	Diphenoxylate	8	453.4	379.3	106	35	9.55				Y
81	Diphylline	2	255.2	181.1	56	30	8.19			Y	Y
82	Diprenorphine	1	426.2	372.4	82	44	8.36				Y
83	Dipyridamol	1	505.4	429.4	118	60	8.80			Y	Y
84	Dobutamine	1	302.2	137.1	68	31	8.04			Y	Y
85	Domperidone	8	426.2	175.2	81	41	8.61			Y	Y
86	Donepzil	8	380.3	243.1	101	40	8.75			Y	Y
87	Dorzolamide	2	325.0	235.9	63	30	7.84				Y
88	Doxapram	2	379.0	292.2	87	31	8.59			Y	Y
89	Doxepin	2	280.0	235.1	74	40	8.92				Y
90	Doxylamine	1	271.2	182.1	35	40	7.92			Y	Y
91	Droperidol	1	380.2	165.1	45	43	8.58		Y	Y	Y
92	Embutramide	8	294.2	191.2	59	27	10.31			Y	Y
93	Eperisone	8	260.4	98.2	51	40	8.83			Y	Y
94	Ephedrine	1	166.3	117.1	45	26	7.84			Y	Y
95	Estazolam	1	295.1	267.0	58	40	10.20			Y	Y
96	Ethopropazine	2	313.1	114.1	61	26	9.17			Y	Y
97	Famotidine	1	338.1	155.1	50	46	7.46			Y	Y
98	Fenoterol	1	304.2	135.0	60	26	7.78			Y	Y
99	Fenspiride	1	261.2	105.2	81	40	8.00		Y	Y	Y
100	Fluphenazine	1	438.2	171.1	80	40	9.32		Y	Y	Y
101	Flurazepam	1	388.2	315.1	63	40	8.83			Y	Y
102	Fluvoxamine	1	319.2	71.0	76	40	9.15		Y	Y	Y
103	Gabapentin	1	172.2	137.1	47	23	7.72				Y
104	Glafenine	1	373.2	281.1	91	40	8.33			Y	Y
105	Glibenclamide	1	494.5	369.0	48	40	11.24				N
106	Gliclazide	1	324.2	127.1	63	26	10.93		Y	Y	Y
107	Glimepiride	8	491.4	352.0	49	40	11.28				Y
108	Glipizide	1	446.5	321.1	38	40	10.45				N
109	Glycopyrrolate	8	318.2	116.1	61	40	8.75		Y	Y	Y
110	Guanabenz	2	231.0	171.9	59	33	8.50			Y	Y
111	Halazepam	2	353.0	241.0	77	40	11.49			Y	Y
112	Haloperidol	1	376.2	165.0	60	40	8.99		Y	Y	Y
113	Hexylcaine	2	262.1	163.0	45	23	8.70			Y	Y
114	Hydralazine	1	161.0	89.0	52	32	7.47				Y
115	Hydroxyalprazolam	1	325.1	297.0	80	40	9.98			Y	Y
116	Hydroxyamphetamine	2	152.2	107.0	31	28	7.60				Y
117	Hydroxymethamphetamine-p	1	166.2	107.0	42	27	7.51			Y	Y
118	Hydroxytriazolam	1	359.1	176.0	76	40	10.03			Y	Y
119	Hydroxyzine	1	375.2	201.1	58	40	9.15		Y	Y	Y
120	Hyoscine	1	304.2	156.1	52	40	7.90			Y	Y
121	Hyoscyamine	1	290.2	124.1	94	32	8.16			Y	Y
122	Imipramine	1	281.0	86.1	41	26	9.07		Y	Y	Y
123	Ipratropium	1	332.2	166.4	51	40	8.17		Y	Y	Y

Table 1 (*Continued*)

No.	Drug name	From sup.	MRM transition parameters				Rt (min)	Detected at spiked level? (ng/ml)[a]			
			Q1	Q3	DP	CE		0.1	1	10	100
124	Isocarboxazid	2	232.2	91.0	54	39	10.05		Y	Y	Y
125	Isoetharine	1	240.2	180.2	51	28	7.56		Y	Y	Y
126	Isoprenaline	1	212.2	194.2	45	17	10				N
127	Isopropamide	1	353.2	238.2	32	22	8.57	Y	Y	Y	Y
128	Isoxicam	1	336.2	99.0	57	24	11.34			Y	Y
129	Isoxsuprine	1	302.1	150.1	47	33	8.50		Y	Y	Y
130	Ketamine	1	238.2	207.2	47	24	8.00			Y	Y
131	Ketotifen	1	310.2	96.1	85	36	8.56			Y	Y
132	Labetolol	1	329.2	162.1	56	40	8.66		Y	Y	Y
133	Lidocaine	1	235.2	86.0	54	24	8.09		Y	Y	Y
134	Lorazepam	1	321.1	275.2	74	32	10.33			Y	Y
135	Lormetazepam	8	335.0	289.2	66	40	10.74		Y	Y	Y
136	Losartan	8	423.1	207.3	71	40	10.42		Y	Y	Y
137	Loxapine	1	328.1	271.0	87	34	8.98		Y	Y	Y
138	Loxoprofen	1	247.3	201.2	77	17	10.43				Y
139	Lysergic acid diethylamide	2	324.2	223.1	65	40	8.58			Y	Y
140	Maprotiline	1	278.2	219.1	60	40	9.16			Y	Y
141	Mepenzolate	1	340.2	130.1	85	41	8.51		Y	Y	Y
142	Mephenoxalone	8	224.1	135.0	46	27	9.55				Y
143	Mephentermine	1	164.1	91.0	33	31	8.09		Y	Y	Y
144	Mepivacaine	1	247.2	98.0	81	31	8.18		Y	Y	Y
145	Meprylcaine	2	236.1	177.3	69	21	8.30			Y	Y
146	Metaproterenol	1	212.2	152.2	51	27	7.46				Y
147	Metergoline	1	404.2	312.2	88	40	9.17				Y
148	Methadone	2	310.0	265.2	50	40	9.17				Y
149	Methaqualone	2	251.1	132.2	71	40	10.57		Y	Y	Y
150	Methdilazine	2	297.2	266.2	67	29	9.09				Y
151	Methocarbamol	1	242.2	118.0	38	16	9.25			Y	Y
152	Methotrimeprazine	2	329.1	100.1	75	28	9.17			Y	Y
153	Methoxyphenamine	1	180.2	121.1	44	18	8.42		Y	Y	Y
154	Methysergide	1	354.2	237.1	93	40	7.91			Y	Y
155	Mexilitine	1	180.2	121.1	35	25	8.42		Y	Y	Y
156	Midazolam	1	326.1	291.1	102	40	8.84	Y	Y	Y	Y
157	Minaprine	1	299.1	212.2	78	28	7.87		Y	Y	Y
158	Modafinil	1	296.0	129.0	48	19	9.70			Y	Y
159	Nabumetone	1	229.1	171.0	62	24	11.31			Y	Y
160	Nalbuphine	8	358.0	272.2	88	41	8.08			Y	Y
161	Nalorphine	1	312.0	201.2	88	39	7.69				Y
162	Naloxone	1	328.2	268.3	57	35	7.73			Y	Y
163	Nicardipine	8	480.3	315.2	66	40	9.15		Y	Y	Y
164	Nicotine	1	163.2	130.2	62	29	4.88			Y	Y
165	Nifenazone	1	309.2	106.0	72	41	8.46		Y	Y	Y
166	Nikethamide	4	179.2	107.9	41	27	8.42		Y	Y	Y
167	Nitrazepam	8	282.1	236.1	81	40	10.33			Y	Y
168	Nordiazepam	1	271.1	139.9	86	40	10.50			Y	Y
169	Norephedrine	3	134.1	91.0	61	41	7.55				Y
170	Nortriptyline	1	264.2	233.2	43	40	9.15			Y	Y
171	Nylidrin	1	299.8	150.1	55	33	8.66		Y	Y	Y
172	Olanzapine	8	313.1	256.2	85	35	7.62		Y	Y	Y
173	Orphenadrine	4	270.3	166.2	43	44	8.92		Y	Y	Y
174	Oxaprozin	1	294.1	234.2	66	31	11.10			Y	Y
175	Oxazepam	1	287.0	241.0	81	40	10.25		Y	Y	Y
176	Oxybutynin	1	358.2	142.2	58	34	9.21			Y	Y
177	Oxycodone	1	316.2	256.2	70	38	7.84			Y	Y
178	Oxyphenonium	1	348.3	132.2	93	43	8.94			Y	Y
179	Panacuronium	1	286.3	236.8	60	22	8.05				Y
180	Pentazocine	2	286.2	218.2	74	40	8.58			Y	Y
181	Pentoxifylline	1	279.3	181.2	60	25	9.12			Y	Y
182	Perindopril	8	369.3	172.0	56	40	8.90			Y	Y
183	Perphenazine	1	404.2	171.1	85	40	9.07			Y	Y
184	Pethidine	8	248.3	220.2	81	28	8.50		Y	Y	Y
185	Phenazone	1	189.0	106.1	66	35	8.98				Y

Table 1 (*Continued*)

No.	Drug name	From sup.	MRM transition parameters				Rt (min)	Detected at spiked level? (ng/ml)[a]			
			Q1	Q3	DP	CE		0.1	1	10	100
186	Phencyclidine	3	244.2	86.1	37	40	9.55		Y	Y	Y
187	Phendimetrazine	2	191.9	148.1	79	32	7.79			Y	Y
188	Phenmetrazine	2	177.9	117.1	57	34	8.96			Y	Y
189	Pindolol	1	249.2	116.1	54	38	8.97				Y
190	Piribedil	1	299.2	135.0	58	37	8.08		Y	Y	Y
191	Piroxicam	1	332.2	95.0	59	43	10.52			Y	Y
192	Prazepam	1	325.0	271.0	77	40	11.58		Y	Y	Y
193	Prilocaine	1	221.2	86.1	46	21	7.99		Y	Y	Y
194	Proadifen	1	354.2	167.2	69	44	9.36		Y	Y	Y
195	Procaine	1	237.0	164.1	54	25	8.88			Y	Y
196	Prochlorperazine	1	374.1	141.1	72	32	9.71			Y	Y
197	Procyclidine	1	288.3	84.1	64	51	8.87		Y	Y	Y
198	Promazine	1	285.2	58.1	76	40	9.63		Y	Y	Y
199	Promazine/promethazine sulphoxide	5	301.3	256.2	65	35	8.29				Y
200	Promethazine	1	285.2	198.1	66	40	9.63				Y
201	Propafenone	1	342.2	116.1	66	33	9.01			Y	Y
202	Propantheline	1	368.2	181.2	51	40	9.63			Y	Y
203	Proparacaine	1	295.1	222.3	53	28	8.32			Y	Y
204	Propionyl promazine	1	341.1	58.0	71	40	9.71		Y	Y	Y
205	Propranolol	1	260.3	116.0	71	40	9.55			Y	Y
206	Protriptyline	1	263.9	233.3	54	23	9.63			Y	Y
207	Quazepam	2	387.0	354.0	100	40	11.89			Y	Y
208	Quetiapine	8	384.2	253.2	86	40	8.81		Y	Y	Y
209	Quinapril	8	439.2	234.1	76	27	9.48			Y	Y
210	Ramipril	8	417.3	234.3	76	40	9.32			Y	Y
211	Ranitidine	1	315.2	176.2	30	28	7.61			Y	Y
212	Reserpine	1	609.4	397.2	80	40	9.40			Y	Y
213	Risperidone	1	411.3	191.3	89	39	8.34		Y	Y	Y
214	Rivastigmine	8	251.2	206.2	66	23	8.35				Y
215	Romifidine	8	258.1	160.2	77	46	7.51			Y	Y
216	Ropivacaine	8	275.2	126.0	36	40	8.44		Y	Y	Y
217	Salbutamol	1	240.1	148.0	60	30	7.64		Y	Y	Y
218	Selegiline	8	188.2	119.1	51	21	8.27			Y	Y
219	Sertraline	8	306.1	159.0	36	40	9.40				Y
220	Sildenafil	8	475.2	283.1	80	43	8.98				Y
221	Sufentanil	2	387.2	238.1	72	40	9.15		Y	Y	Y
222	Sulpiride	1	342.2	112.1	75	40	7.66		Y	Y	Y
223	Suxibuzone	1	439.2	321.2	57	20	11.13				Y
224	Telmisartan	8	515.3	276.1	101	71	9.48			Y	Y
225	Terazocin	8	388.3	71.0	96	40	8.27			Y	Y
226	Terfenadine	1	472.4	436.4	78	36	9.55			Y	Y
227	Tetracaine	1	265.3	176.2	34	27	8.72		Y	Y	Y
228	Tianeptine	1	437.1	292.2	48	26	8.91		Y	Y	Y
229	Timolol	1	317.1	261.0	80	40	8.27				Y
230	Tolbutamide	1	271.2	155.0	68	26	10.61			Y	Y
231	Tolmetin	1	258.1	119.0	51	27	10.71		Y	Y	Y
232	Tramadol	8	264.2	58.0	51	40	8.35		Y	Y	Y
233	Trihexyphenidyl	1	302.3	98.1	45	30	8.95		Y	Y	Y
234	Trimipramine	1	295.1	100.1	63	27	9.27			Y	Y
235	Triplennamine	1	256.2	211.1	44	33	8.43		Y	Y	Y
236	Valsartan	8	436.2	207.4	21	37	11.08			Y	Y
237	Vardenafil	8	489.2	312.2	126	60	8.70				Y
238	Verapamil	1	455.2	165.1	91	40	9.19			Y	Y
239	Xylazine	1	221.1	90.0	69	35	8.35			Y	Y
240	Yohimbine	1	355.3	144.1	46	40	8.51		Y	Y	Y
241	Zomepirac	1	292.2	139.0	53	27	11.03			Y	Y
242	Zopiclone	1	389.2	245.1	44	25	8.19			Y	Y
243	Zuclopenthixol	8	401.2	221.0	51	75	9.27				Y
	Subtotal							4	83	199	239
	Percentages per spiking level (A)							2	34	82	98

Table 1 (*Continued*)

No.	Drug name	From sup.	MRM transition parameters				Rt (min)	Detected at spiked level? (ng/ml)[a]			
			Q1	Q3	DP	CE		0.1	1	10	100
(b) MRM acquisition parameters and the limit of detection achieved for the target analytes screened using MRM exp 2											
244	4-Methylthioamphetamine	3	182.1	137	32	26	8.2	NDT	NDT	NDT	Y
245	Benzoylecgonine	3	290.2	77	71	40	8.59	NDT	NDT	NDT	N
246	Clomipramine	1	317.1	86	61	40	9.19	NDT	NDT	NDT	Y
247	Clonazepam	2	316.1	270	68	40	10.29	NDT	NDT	NDT	Y
248	Detomidine	8	187	81	47	24	8.29	NDT	NDT	NDT	Y
249	Eltenac	8	301.8	284	55	18	11.32	NDT	NDT	NDT	N
250	Fenfluramine	1	232	159.1	49	30	8.53	NDT	NDT	NDT	Y
251	Fentanyl	8	337.2	188.1	67	40	8.62	NDT	NDT	NDT	Y
252	Indoprofen	1	282.1	236.1	77	32	10.32	NDT	NDT	NDT	Y
253	Methazolamide	1	239.2	89	74	17	8.09	NDT	NDT	NDT	N
254	Moclobemide	8	269.2	182.1	51	40	8.06	NDT	NDT	NDT	Y
255	Pipenzolate	1	354.1	144.1	46	40	8.56	NDT	NDT	NDT	Y
256	Temazepam	2	301	255.1	58	40	10.52	NDT	NDT	NDT	Y
257	Trifluoperazine	1	408.1	141.1	70	33	9.21	NDT	NDT	NDT	Y
	Subtotal							–	–	–	11
	Percentages per spiking level (B)							–	–	–	79
	Total							4	83	199	250
	Percentages per class (Overall)							2[c]	34	82	97

[a] N: drugs not detected or intensity < threshold, Y: drugs detected and NDT: not determined.
[b] Denotes the internal standard.
[c] These percentages do not include those drugs that were not analysed at the lower concentration levels.

Asia, Singapore) Oasis HLB® (2.1 mm × 20 mm, 25 μm) and Sunfire® (2.1 mm × 150 mm, 5 μm) columns were installed into the flow path as shown in Fig. 1. The autosampler, plumbed so that needle could be back-flushed by the quaternary pump, was used in a programmed mode to load the extended loop three times with the volume entered into the Analyst sample batch list. After an additional volume was drawn up and the needle returned to the seat, the valve switched to mainpass position and the run was triggered. The total amount injected was 400 μl.

2.2.1. The settings for the LC pumps

2.2.1.1. Quaternary pump.

Agilent HP1100						
Step	Time (min)	Flow-rate (μl/min)	A (%) 0.15% formic acid water	B (%)	C (%) acetonitrile	D (%) 10 mM ammonium acetate pH10
0	00.00	2000	0	Not used	0	100
1	00.40	2000	0		0	100
2	00.50	2000	100		0	0
3	00.80	2000	100		0	0
4	00.90	100	100		0	0
5	01.00	100	100		0	0
6	08.25	300	0		100	0
7	12.00	500	0		100	0
8	12.50	500	0		100	0
9	13.00	500	100		0	0
10	14.00	500	100		0	0

2.2.1.2. Isocratic pumps.

Step	Pump 1: Agilent HP1100		Pump 2: Shimadzu LC6A	
	Time (min)	Flow-rate μl/min (water)	Time (min)	Flow-rate (μl/min) (95:5 water:methanol)
0	00.00	0	0.00	300
1	01.00	400		
2	08.25	200		
3	12.00	0		
4	12.50	0		
5	12.60	2000		
6	13.50	2000		
7	14.00	0	14.00	300

2.2.2. The settings used for the 4000 Q TRAP

TIS at 500 °C was used with a curtain gas 20 psi, nitrogen collision gas (CAD) set to high, GS1 35 psi and GS2 45 psi. The current was 3 kV with the interface heater on. The entrance potential was set to 10 for all transitions. An intensity threshold of 250 cps was set for MRM experiment 1 (Q1 and Q3 unit resolution) and 1000 cps for experiment 2 (Q1 and Q3 high resolution), declustering potential (DP), collision energy (CE) plus other instrumental parameters used for the MRMs are shown in Table 1. The IDA criteria were set to acquire an EPI spectrum (maximum fill time 20 ms) when the signal was greater than 1000 cps for the most intense peak on the inclusion list within the corresponding time window. Former target ions were always excluded and an exclusion list was used to avoid EPI being prematurely triggered before the time window in which the drug was expected to appear. The Dynamic Background Subtraction settings used were an average of 1 background subtraction and 5 data points smooth.

2.2.3. The settings used for the switching valves

Time	Switching valve		
	6 Port (column compartment)	Valco 10-port valve #1	Valco 10-port valve #2
0.00	1 → 2 (T-piece to 10 port valve #1) 3 → 4 (autosampler to ext. col)	5 → 6 (Iso. pump. 2 to 10-port valve #2) 7 → 8 (8 to waste)	5 → 6 (10 port valve #1 to analytical col. and TIS)
1.00	1 → 2 3 → 4	7 → 6 (to 10 port valve #2)	5 → 6
12.5	1 → 6 (T-piece to flow path blocker) 3 → 2 (autosampler to 10 port valve #1) 4 → 5 (ext col. To waste)	7 → 6	5 → 6
14.0	1 → 2 3 → 4 5 → 6	7 → 6	5 → 6

2.3. Sample preparation

The urine was adjusted to pH 4.75 ± 0.1 before 900 μl of sample was transferred into an Axygen (Union City, CA, USA) 1.5 ml plastic micro-sample tube with the addition of 20 μl of the internal standard (D_3-codeine glucuronide) at a concentration of 10 μg/ml. After the addition of 20 μl of the *Helix pomatia* β-glucuronidase/aryl sulphatase enzyme and incubation at 55 °C for 2 h, 100 μl of methanol was added to the sample and it was mixed by repeated drawing up and expulsion of the liquid back into the tube. The tubes were centrifuged in a Heraeus Biofuge® Pico at an RCF of 16,000 × *g* for 10 min. Supernatant was transferred into an Axygen (Union City, CA, USA) 96 well plate.

2.4. Method validation

Primary stock solutions (PSS) of all the reference standards used were prepared at a concentration of either 1 or 0.1 mg per ml of methanol. Five groups of drugs A (50), B (50), C (50), D (51) and E (56) PSS were combined to achieve a working stock solution concentration of 1000 nanograms per ml for each of the targeted drugs. Blank equine urine, taken from the laboratory's negative post-race samples, was spiked with 100 ng equivalent of all drugs in each group. The spiked urine was serially diluted 1:10 with blank urine to achieve a concentration of 10, 1 and 0.1 ng/ml. Three aliquots of each group spiked samples, along with three samples of the urine used to prepare the spiking (urine blank), were processed as shown above. Three intra-assay injections were made for each of the samples and the drug was considered as detected if its MRM peak area was >5e2 within the corresponding retention time (RT) window for the authentic reference standard.

2.5. Thoroughbred racehorse drug administration

Samples collected from thoroughbred racehorses and distributed under the Asian Quality Assurance Positive Programme of the Association of Official Racing Chemists were supplied to the laboratory by the following organizations:

2.5.1. Propantheline bromide 300 mg - Racing Science Centre (Brisbane, Australia)

Propan B (Glenorie, NSW, Australia) was administered to two 500 kg geldings by IV and IM respectively and urine samples were collected for 48 h after administration. The collected post-administration urine was pooled and frozen before the aliquots of the pre- and post-administration were dispatched by courier to Singapore.

Modafinil 500 mg—Australian Racing Forensic Laboratory (Sydney, Australia) was administered with a small amount of feed to a 550 kg gelding, the urine was collected and stored as described in [1]. The collected post-administration urine was pooled and frozen before the aliquots of the pre- and post-administration were dispatched by courier to Singapore.

3. Results and discussion

Infusion of our reference standards into the DuoSpray® source allowed us to assign them into groups based on the ionisation mode that produced the largest relative abundance for each one. We found that positive TIS gave the best response for the majority of our drug standards and, for that reason, this was the first group for which we developed an analytical method.

The starting point for our LC–LC method development was the selection of the Waters Oasis HLB® polymeric extraction cartridge. This decision was primarily based upon its demonstrated ability to perform under direct-injection conditions (e.g. [21–23]) and, more importantly, the characteristics of the polymer were well suited to extracting drugs of widely differing chemistry. The latter consideration was of critical importance, since the method we describe here was being developed in conjunction with analyses designed to target acidic and neutral drugs using the same extraction platform. Three software controlled sample switching valves were employed to stream un-retained material to waste, achieve the differential gradient between extraction and analytical columns and back-flush particulate matter off the first in-line filter after the extraction step was completed.

The LC configuration was selected after a preliminary study, using an organic solvent gradient to elute drugs from an extrac-

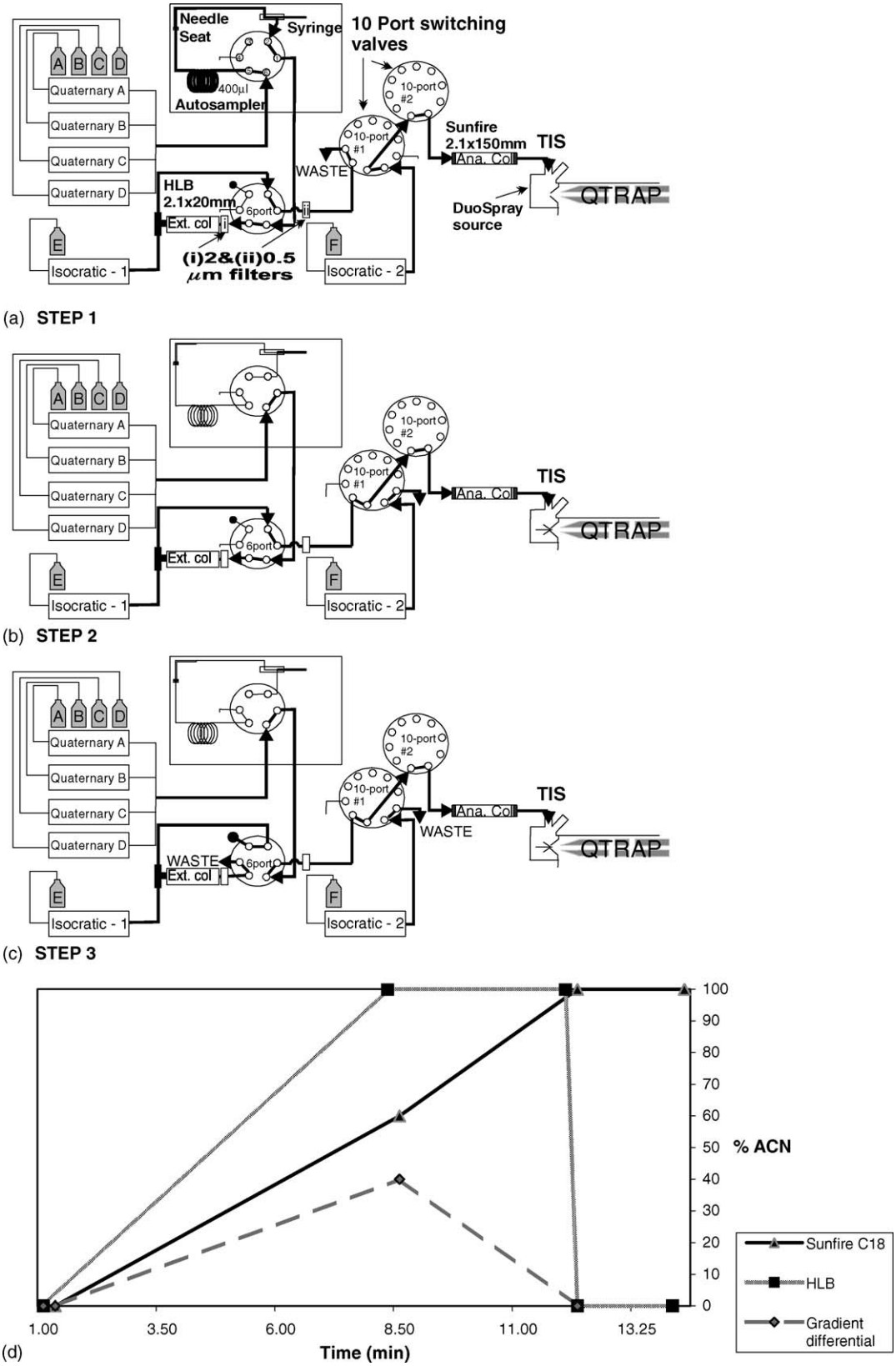

Fig. 1. (a) Transfer of sample from loop to the extraction column, (b) analyte elution, refocusing and separation using differential gradient chromatography LC–LC, (c) back-flushing of the extraction cartridge plus completion of the elution from analytical column and (d) graph showing elution gradients for extraction and analytical columns and the gradient differential.

tion cartridge directly onto an analytical column, gave broad peaks that were very similar in appearance to those observed when using the extraction cartridge alone. Hence, we speculated that the polymeric HLB® packing material used was more retentive of the analytes than the C18 stationary phase under these conditions and the percentage acetonitrile that effected elution of a particular drug from the polymer particles was sufficient to subsequently retard or even prevent absorption of the same drug onto the silica-based packing material. Once we experimented with the dilution of the first column eluent with water, we were able to achieve the desired resolution and analyte peak shape with the columns in series. Therefore, we configured our liquid chromatographic system to provide the capability for the eluent from the extraction cartridge to be proportionally diluted with water in a controlled manner (see Fig. 1) to produce a linear organic solvent gradient at different rates across the two columns. Another advantage to this set up was that it allowed for high flow back-flushing of the cartridge in the terminal part of the run and this assisted with the removal of any particulate material trapped on the filter unit's frit. Furthermore, because of the way in which the system has been configured, it can also be used in the normal mode (i.e. bypass the direct injection set-up) without having to make any modification to the flow path plumbing.

The applicability of the differential LC–LC gradient (see Fig. 1d) for separating the selected drugs was evaluated by analysing urine samples spiked at 100 ng/ml. Analysis of the MRM data showed that the analytes had an average peak width (FWHH) value of 0.19 min and an asymmetry value (10% peak height) between 0.9 and 1.3. We considered these values acceptable for our work.

At the highest concentration spiking, two hundred and fifty three of the 257 target analytes gave a MRM response that exceeded our detection criteria (RT ± 0.2 min. + >5e2 peak area) when using an experiment with a threshold of 250 cps and unit resolution on both Q1 and Q3. However, for 14 of the drugs detected, we also observed that the urine blank had interference peaks that fell within the expected retention time window. Therefore, we elected to move these drugs' MRM transitions into a second experiment using Q1 and Q3 at the high-resolution setting and increased the threshold to 1000 cps. This change eliminated the co-eluting peaks from the blank urine; however, this also led to the failure to detect three of the fourteen drugs at the 100 ng/ml spiked level. The limits of detection, in the MRM mode, that were determined for all the drugs analysed by experiment 1 and 2 are shown in Table 1a and b, respectively and it is notable that, in many cases, these are lower than those we have achieved using commercially available enzyme linked immunosorbent assay kits. Those that are shown as not detected in Table 1 were dropped from this method and their detection using other separation and/or ionisation conditions will be evaluated at a later stage.

One of the strengths of the hybrid tandem mass spectrometer is the ability to use software to switch the operational mode from quadrupole-quadrupole (QqQ) MRM to quadrupole-linear ion trap (QqLIT) to acquire enhanced product ion spectra when using the data dependent scan mode. Other equipment manufacturers have offered this scanning mode on their triple quadrupole and three-dimensional ion trap mass spectrometers. However, the 4000 QTRAP has the advantage over the triple quadrupole (QqQ) as switching to the (QqLIT) mode enhances sensitivity [25] for the acquisition of product ion spectra. It is also widely accepted that three-dimensional ion traps are not well suited to undertaking survey scans, as the sensitivity is severely compromised whenever multiple mass reaction monitoring experiments or other survey scans are included in the experiment. Therefore, the hybrid mass spectrometer is superior, as it meets the requirements of performing both the survey and dependent scanning steps with adequate sensitivity to undertake data dependant scanning effectively.

The most effective way to make use of this capability is to employ rapid QqQ MRM survey scans to continuously sample the eluent to establish if any of the target analytes are present and only trigger the more time consuming QqLIT EPI scan once a target signal exceeds the defined IDA criteria. This data dependant acquisition arrangement provides collateral data that can help establish the presence of the drug to be acquired within the same run. For example, Fig. 2a shows the MRM survey scans for midazolam and b shows the XIC for the dependant EPI scans triggered by urine spiked with this drug at 0.1 ng/ml.

The application of dynamic background subtraction (DBS) to enhance the operation of the IDA mode has been published [26] and, in our hands, proved more effective than when the method was applied without using this script. Nonetheless, analysis of urine samples spiked with groups of approximately 50 analytes at 1 ng per ml showed, that when we use DBS plus IDA, approximately 33% of the drugs that met the defined threshold criteria still did not trigger an EPI scan. Even taking into account that it was possible that competition between the 50 or so drugs in each group interfered with the functioning of the IDA step, it was obvious to us that the screen might be unreliable if it was based solely upon checking the dependent experiment data. For this reason, we elected to use the Analyst® quantitation and query wizard to flag MRM peaks that were within the expected retention time window and above a defined intensity. This list was manually crosschecked against the IDA generated data and, if the software had failed to trigger the required product ion scan, the sample was reanalysed using a non-IDA method to obtain the desired EPI.

The EPI spectra obtained from 0.1 and 100 ng/ml spiked urine sample are shown in Fig. 2c and d, respectively. However, one of the drawbacks of the Analyst® data dependant scan mode is the reliance upon using a single pre-selected CE or collision energy spread (CES) setting for all the analytes. When we used a CES of 40 ± 25 V, some analytes underwent limited fragmentation and there were others where fragmentation was so extensive that no usable spectrum was obtained. It has been proposed [24] that carrying out three separate EPI scans at a high, medium and low fixed CE can overcome this limitation, however this increases the duty cycle and we found that this significantly decreased sensitivity. Instead, we adopted the lower CES range of 20 ± 10 V, which gives averaged spectra from CE at 10, 20 and 30 V within a single scan. However, these lower collision energy settings produced some (<10%) spectra that had too few

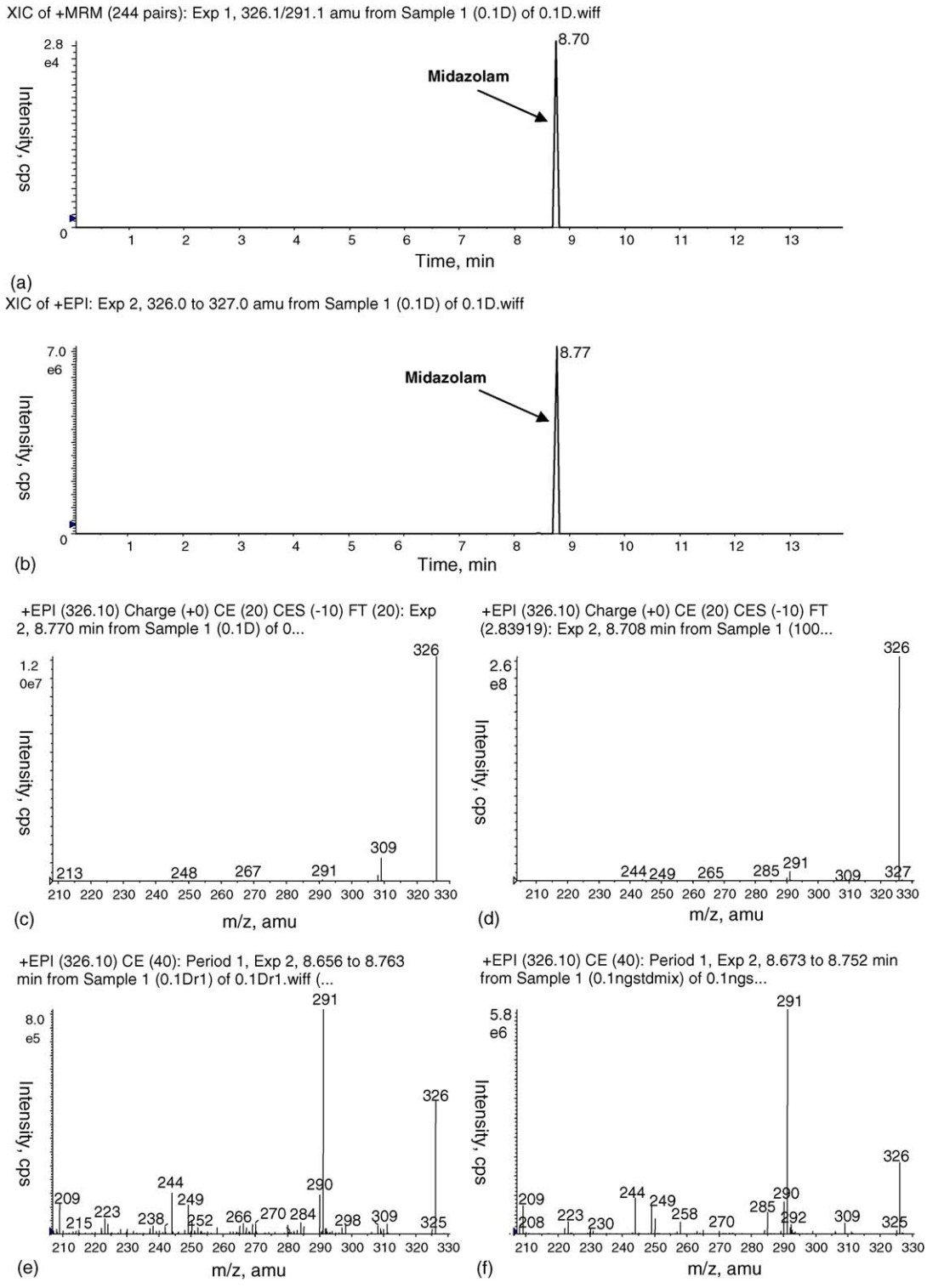

Fig. 2. Extracted ion chromatogram: (a) MRM 326.1 > 291.1 AND (b) enhanced product ion chromatogram 326.1 from midazolam 0.1 ng/ml spiked urine. The CES 20 ± 10 V EPI spectrum of the 0.1 ng/ml and 100 ng/ml spiked sample are shown in (c) and (d) respectively and the EPI at CE 40 V for the spiked sample is shown in (e) and standard at 0.1 ng/ml, acquired under the same conditions, is shown in (f).

product ions to be able to unequivocally declare that there was an acceptable qualitative match to the standard. This is illustrated by the data obtained from midazolam, where the EPI spectra obtained using the IDA CES setting (Fig. 2c and d) were dominated by the still intact precursor ion. Whereas a fixed CE 40 V spectrum (Fig. 2e) from the 0.1 ng/ml sample contained several significant fragment ions that would be of great assistance when trying to determine whether an unknown spectrum was a true match to the standard (0.1 ng/ml at 40 V, Fig. 2f). Feedback from the instrument's manufacturer is that future releases of Analyst®

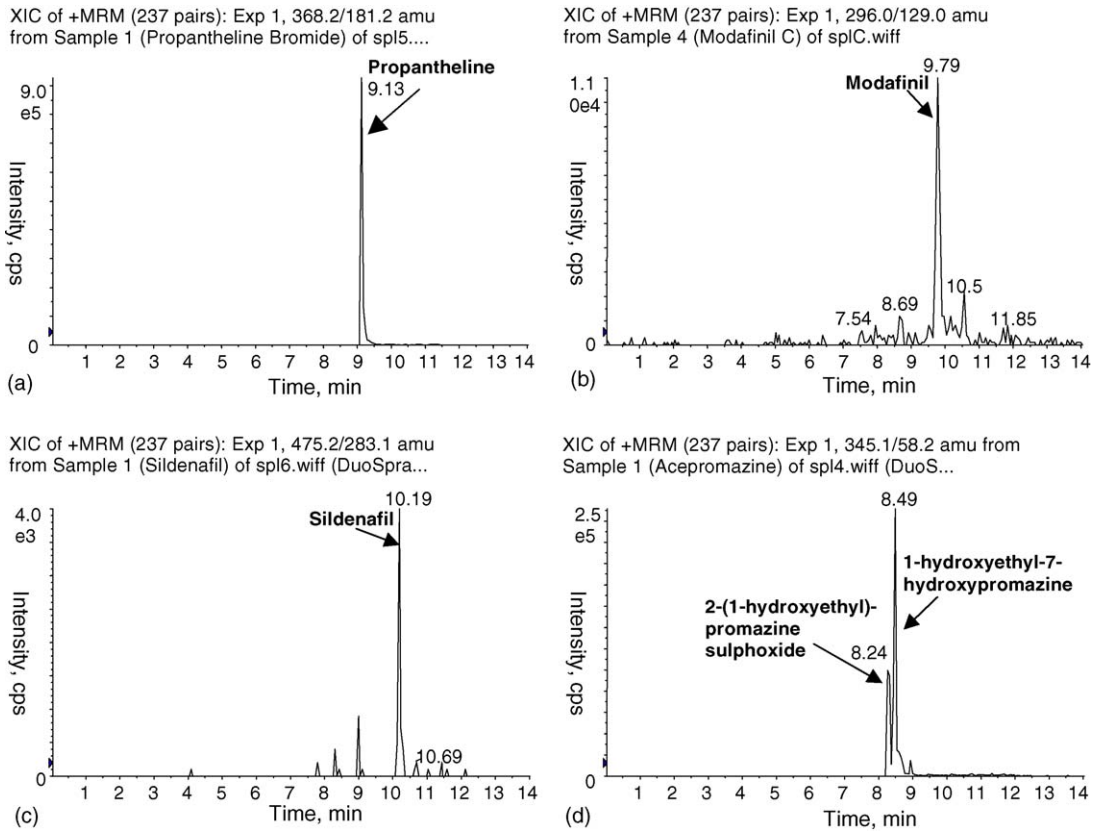

Fig. 3. Extracted MRM ion chromatograms of: (a) 368.2 > 181.2 from analysis of 8 h. post 300 mg propantheline bromide administration equine urine and (b) 296.0 > 129.0 (sodium adduct) from analysis of 24 h. post 500 mg modafinil administration equine urine. The extracted ion chromatograms (c) 475.2 > 283.1 and (d) 345.1 > 58.2 are from analysis of positive samples previously shown to contain the drugs sildenafil and acetylpromazine respectively.

software may allow the individual CE and DP settings from the MRM to be ported for use by the EPI dependant scan mode. This approach should rectify this issue and lead to the generation of EPI spectra with the optimum amount of fragmentation for each drug. Nonetheless, until this becomes available, we will have no other options but to re-screen suspect hits using a fixed CE method whenever the IDA generated EPI spectrum shows a limited number of fragment ions.

The final stage of data checking involved comparison of the EPI spectrum from any flagged peak with that obtained from analysis of an authentic reference standard. We undertook tedious and time consuming manual spectral comparison even though Analyst® 1.4.1 has library-searching capabilities included. This was because the discriminant ability of the current programme appears compromised, as spectra comprised of a significant proportion of low mass to charge product ions gave unduly poor matches and others, with limited fragmentation of the precursor ion, always returned a high match value. Based upon these observations, we speculate that this algorithm is more suited to searching single quadrupole electron impact data than MS/MS spectra. Hopefully this issue will also be resolved in future upgrades, as undertaking semi-manual data checking limits either the number of samples that can be screened or total drug targets per analysis, to a level that can be routinely achieved within the reporting timeframe. With this constraint removed, there is an option to distribute the MRM scans into two or, as the retention time difference between the fastest and slowest eluting drug was 7 min, possibly more time segments. Using multiple segments with $\leq$250 concurrent pairs will allow the total number of analytes to be expanded while, at the same time, sensitivity will not be lost.

The ability of our method to correctly identify a drug in a sample from a treated horse was evaluated by analysing aliquots of urine collected after administration of known quantities of either modafinil (500 mg) or propantheline bromide (300 mg). We also analysed two post-race urine samples previously declared positive to either acepromazine or sildenafil and these provided a useful gauge of the suitability of the method for detecting these substances at concentrations typically encountered in doping surveillance. The extracted ion chromatograms are shown in Fig. 3a–d and demonstrate that the drug or its metabolites were detectable in all four samples.

Apart from its applicability as a screening method, the system can also be used in the non-IDA mode to provide legally defensible confirmatory analytical data suitable for proving that the drug was present in a sample. For example, Fig. 4a shows the product ion spectrum obtained using the EPI scan mode (without IDA) to obtain products of the molecular ion in the propantheline post-administration urine sample. This is a very good match with the spectrum (Fig. 4b) obtained from the analysis of an authentic reference standard and, combined with fact that the LC retention time matches, confirms

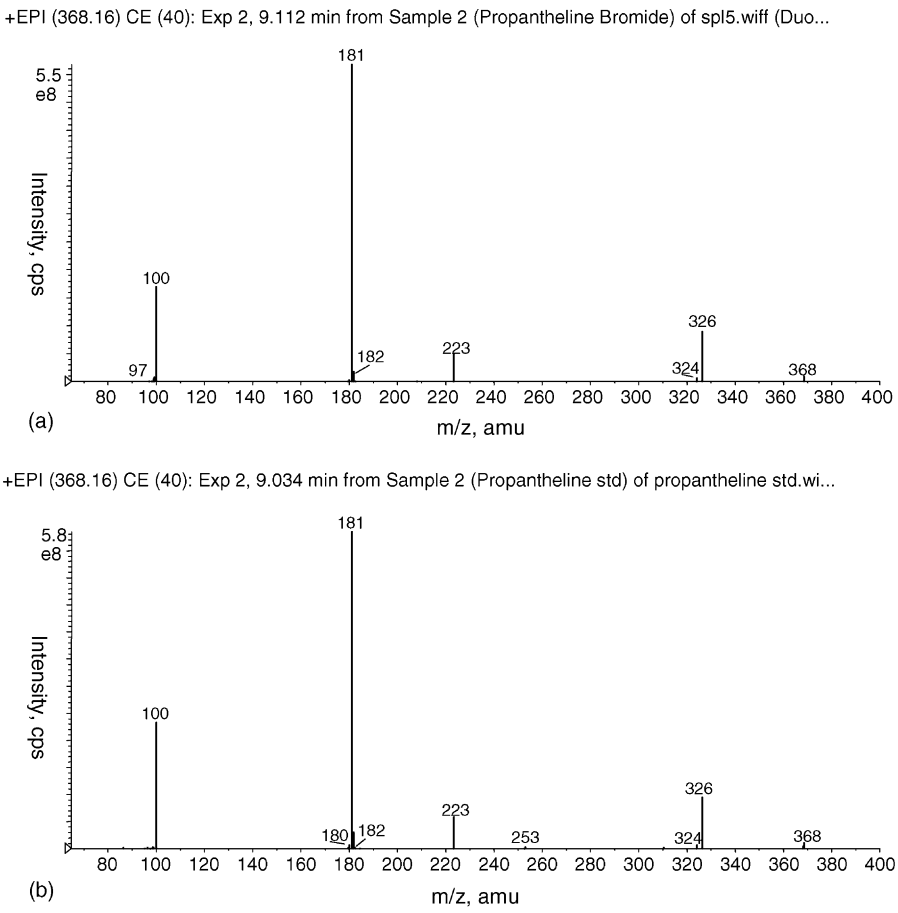

Fig. 4. Enhanced product ion (*m/z* 368.16) spectrum from: (a) analysis of a urine sample from horse administered 300 mg propantheline bromide and (b) authentic propantheline reference standard.

the presence of the drug in the sample using two independent techniques.

The method has proved to be robust and blockages were infrequent if the frits in the in-line filter units were exchanged after every 200 injections of urine. In our experience, the downtime frequency rate is comparable to that which we have experienced when using off-line sample preparation coupled with conventional LC and we did not find that the extraction and analytical columns required changing on a very frequent basis. The influence of the urine matrix upon the LC retention time stability has been investigated and we have found that the percentage inter-assay coefficient of variation for standards, spiked in three separate batches of urine, was less than 0.3%. When this result is considered in conjunction with the acceptable retention time match ($< \pm 0.2$ min.) achieved between the drugs when spiked into water and when spiked into various samples of negative post-race equine urine, we concluded that the retention time reproducibility is adequate for our purposes.

4. Conclusion

The method we have developed can rapidly screen for a large number of basic drugs using a small volume of enzyme-hydrolysed equine urine. Since there is no pre-extraction step where analytes could be lost, the only drugs that cannot be targeted using this method are those that do not bind to the HLB extraction cartridge, those that bind strongly/irreversibly to either of the LC columns and those that do not give a suitable response under TIS MS/MS conditions. Our results showed that this affected a very small (<3%) proportion of the basic drugs that we targeted. The method is economical in terms of sample usage and consumed approximately 1.6 μl per target analyte. Moreover, it is also cost effective because of the reduced labour requirements and the consumable expenses per sample are less than encountered with off-line sample extraction methods. For example, we were able to use each HLB® extraction cartridges for more than 1000 urine sample injections without impacting on the system performance. Therefore, even though on-line extraction cartridges are approximately 150 times more expensive than the corresponding SPE cartridges, the unit cost per sample is much lower.

The drawback of the method is the tediousness of the data-checking steps and this has prevented us from increasing the number of analytes screened. However, once the chemometric ability of the software catches up with the detection capabilities of the method, we believe that there is still considerable scope for increasing the number of target analytes. In fact, we remain confident that sometime in the near future, either the instrument's manufacturer or an independent software developer will provide the data checking capability we require. Once this is done, we

project that the total number can be increased to 500 or more screening targets per sample injection.

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